Theor Appl Genet (1992) 84:688–692

THEORETICAL AND APPLIED GENETICS

Genetic relationships in the genus *Cicer* L. as revealed by polyacrylamide gel electrophoresis of seed storage proteins

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Received August 25, 1991; Accepted December 19, 1991
Communicated by G. S. Khush

Summary. Total seed storage protein of the cultivated chickpea, *C. arietinum* L., and eight other wild annual *Cicer* species (all $2n=16$) was separated and compared by sodium dodecyl sulphate polyacrylamide gel electrophoresis. The seed-protein profile was a conservative and species-specific trait. Relative interspecific similarities of protein patterns were estimated using Jaccard's similarity index, and a cluster analysis was performed. The resultant dendrogram generally agreed with the limited data already available on interspecific relationships in *Cicer* based on morphological characteristics, crossability, genome pairing in hybrids, karyotypes and isozyme analysis. The difference between the profiles of *C. judaicum* and *C. pinnatifidum* supported the idea that they are indeed two separate species. The closest relative of *C. arietinum* was *C. reticulatum*, followed by *C. echinospermum* and other species, while *C. cuneatum* was the farthest relative. In general, *C. cuneatum* was also genetically the farthest removed from any other species. The suggestion that *C. reticulatum* is the wild progenitor of the cultivated chickpea was therefore further supported.

Key words: *Cicer* – Chickpea – Seed storage protein – Electrophoresis – Species relationships

Introduction

The genus *Cicer* L. is best known by the cultivated species *C. arietinum* L. (chickpea), which is an important grain legume in many Old World countries (van der Maesen 1972). There are 42 wild *Cicer* species of which 8 are annual and all except 1 of the remaining 34 (1 has not been classified) are perennial (van der Maesen 1987).

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Several wild species of *Cicer* possess disease resistance and other characters of value in chickpea improvement (van der Maesen and Pundir 1984). A clear understanding of the genetic relationships among various species is essential for successful and efficient utilization of the genetic variability present in the related wild species (Kimber 1984). Such information is lacking in the genus *Cicer* because interspecific hybrids are difficult to produce (Ladizinsky and Adler 1976a; Pundir and van der Maesen 1983; Ahmad 1988) due to strong post-fertilization barrier(s) (Ahmad et al. 1988b).

The basic criterion of phylogenetic relationships is gene homology, which in many cases cannot be measured directly because of reproductive barriers between species (Kimber 1984). The fractionation of "non-essential" seed storage protein (Margoliash and Fitch 1968) by polyacrylamide gel electrophoresis (PAGE) is used as an additional tool for assessing species relationships in many major crop plants (Ladizinsky and Hymowitz 1979; Sammour 1989). Such information supplements evidence from comparative morphology, breeding experiments and cytogenetic analysis of interspecific hybrids.

In the cultivated chickpea, water-soluble (albumin) and electrolyte-soluble (globulin) fractions constitute approximately one-third and two-thirds, respectively, of the total seed storage protein (Vairinhos and Murray 1983). Vairinhos and Murray (1982, 1983) studied the albumin and globulin fractions in three annual *Cicer* species, whereas Ladizinsky and Adler (1975) studied only the albumin fraction in seven of the nine annual *Cicer* species. To date, total seed storage proteins of the nine annual *Cicer* species have not been studied. Therefore, the study presented here was undertaken with the objective of using PAGE profiles of total seed storage proteins to further elucidate genetic relationships among the annual species of *Cicer*.

Materials and methods

Mature dry cotyledons were collected from plants of *Cicer* spp. grown under similar conditions. The species investigated were the cultivated chickpea *C. arietinum* (15 accessions) and eight species of wild annual *Cicer*: *C. bijugum* Rech. (6 accessions), *C. chorassanicum* (Bge.) M. Pop. (2 accessions), *C. cuneatum* Rich. (1 accession), *C. echinospermum* Dav. (2 accessions), *C. judaicum* Boiss. (4 accessions), *C. pinnatifidum* J. and S. (2 accessions), *C. reticulatum* Lad. (6 accessions) and *C. yamashitae* Kit. (2 accessions). The 15 cultivated chickpea accessions originated from India, Iran, Spain, USA, Mexico and Turkey and represented a considerable portion of the global chickpea growing area. The wild annual species represented most of the accessions available from the world germ plasm collection.

Protein extraction was done by homogenizing finely ground cotyledon meal in 0.1 *M* TRIS-HCl buffer (pH 7.5). Samples of the clear supernatant, obtained after centrifugation at 15,000 rpm (15,600 *g*) at 4 °C for 15 min, were diluted with sample buffer and heated in boiling water for 5 min prior to electrophoretic fractionation. Electrophoresis was carried out in the modified discontinuous sodium dodecyl sulfate PAGE (SDS-PAGE) system of Laemmli (1970) using 10% acrylamide separating gel (0.375 *M* TRIS-HCl buffer, pH 8.8) and 4.5% acrylamide stacking gel (0.125 *M* TRIS-HCl buffer, pH 6.8). The electrode buffer was TRIS-glycine (2.25 g TRIS and 10.8 g glycine per 750 ml buffer solution, pH 8.3) with 0.1% SDS. Staining of the gels was done overnight in 0.1% Coomassie Brilliant Blue-R250 solution containing 40% methanol and 10% trichloracetic acid, while destaining was done in the same solution but without the dye. The molecular weight of the migrated polypeptides was determined using the following known molecular weight standard polypeptides: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100) and lactalbumin (14,000). Molecular weight estimates were based on many independent determinations and expressed to the nearest appropriate 1,000 or 500 daltons (Da).

Depending upon the presence or absence of bands, Jaccard's similarity index (S) (Sneath and Sokal 1973) was calculated for all possible pairs of species by the following formula:

$$S = \frac{W}{A + B - W}$$

where W = the number of bands of common mobility, A = the number of bands in species "A" and B = number of bands in species "B". The similarity matrix thus obtained was converted to a dissimilarity matrix (Dissimilarity = 1 – Similarity) and used to construct a dendrogram by the unweighted pair group method of cluster analysis using arithmetic means (UPGMA) (Sneath and Sokal 1973).

Results and discussion

The electrophoretic seed protein profiles of all 15 accessions of the cultivated chickpea were the same, both in number of bands and in their distribution on the gel, even though they represented a wide range of morphological variation. Occasionally, variation was observed in the density or sharpness of a few bands. This uniformity of seed-protein profile agrees with the findings of Ladizinsky and Adler (1975) who examined 88 different cultivars

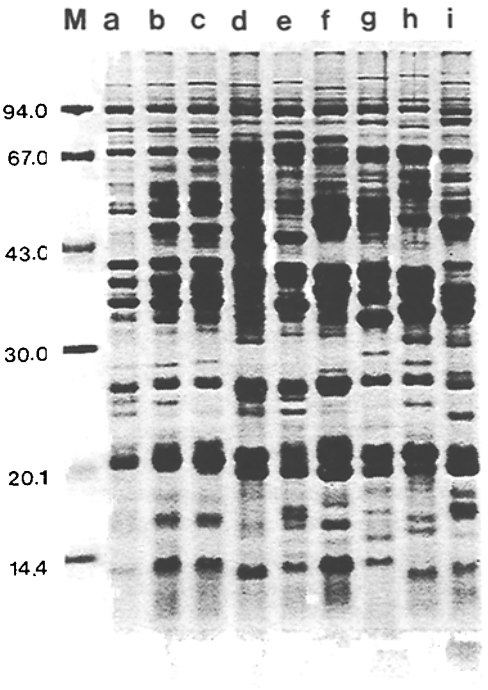

Fig. 1. Electrophoregram showing seed storage protein banding pattern in the annual *Cicer* species. Lane identification is as follows: *a C. arietinum, b C. reticulatum, c C. echinospermum, d C. pinnatifidum, e C. judaicum, f C. bijugum, g C. chorassanicum, h C. yamashitae, i C. cuneatum, M* molecular weight markers (kDa)

of the cultivated chickpea, which represents its entire distribution area, and concluded that seed protein was a very conservative trait in chickpea. The electrophoretic seed-protein profile was also uniform among accessions within the remaining eight wild annual *Cicer* species. Thus, the seed-protein profiles of the nine annual *Cicer* species is largely species-specific.

Eighty-three polypeptide bands, ranging in molecular weight from 9.5 kDa to 98.0 kDa, were identified in the nine annual *Cicer* species (Fig. 1). The maximum number of bands (37) was present in *C. chorassanicum, C. cuneatum, C. judaicum* and *C. pinnatifidum*, while *C. yamashitae* exhibited the minimum number of bands (32). Thirty-five bands were observed in *C. arietinum* and *C. reticulatum*, while *C. bijugum* and *C. echinospermum* showed 33 bands each. Polypeptides having an approximate molecular weight of 20, 36 and 71 kDa were present in all nine *Cicer* species, albeit in different concentrations. Eight species exhibited polypeptides of about 13, 40 and 76 kDa, while bands having an approximate molecular weight of 21, 25, 34, 38, 52, 80, 92 and 98 kDa were observed in seven of the nine *Cicer* species. Certain unique polypeptides were also observed in some species. These included polypeptides of molecular weights 13, 31, 61 and 96 kDa in *C. chorassanicum*, 9.5, 29, 70, and 81

kDa in *C. cuneatum*, 14, 44 and 95 kDa in *C. judaicum*, 93 kDa in *C. pinnatifidum*, 55 kDa in *C. reticulatum* and 22, 37, 53, 79 and 97 kDa in *C. yamashitae*.

In the cultivated chickpea, the major polypeptides of molecular weights 14, 21, 36, 37, 41 and 73 kDa represent legumin and vicilin, the main seed storage proteins (Vairinhos and Murray 1982, 1983). Many of these polypeptides, and a few others, were observed in the present study and must be representative of the polypeptides found in the major seed storage proteins. *Cicer reticulatum* and *C. echinospermum* had major polypeptide profiles very similar to that of *C. arietinum* (Fig. 1). Similar observations were also made by Vairinhos and Murray (1982, 1983). The remaining six *Cicer* species had quite different major polypeptide profiles, not only in comparison with the cultivated chickpea, but also among themselves (Fig. 1). Variation in the major polypeptide profiles indicates substantial differences in amino acid composition, and represents genetic differences among the species (DeJong et al. 1978).

The polypeptide profile for each of these nine *Cicer* species was species specific. Such a constant and unique pattern of proteins is probably the consequence of a species-specific gene arrangement, structure and activity, as found previously in *Vicia* (Ladizinsky 1975; Sammour 1989) and *Lens* (Ladizinsky 1979). In general, the electrophoretic mobility of isozymes (Duke and Glassman 1968) and proteins (Siddiq et al. 1972; Panda et al. 1986) is reduced as species evolve. If this is true, then all nine *Cicer* species must be primitive since many polypeptides of relatively high mobility were observed (Fig. 1). The suggestion that *Cicer* species are indeed primitive is supported by the primitive nature of their karyotypes (Ahmad et al. 1987a; Ahmad 1988) and pollen morphology (Ahmad 1988). A 9.5-kDa polypeptide was present only in *C. cuneatum*, suggesting that this particular species may be more primitive than the other annual *Cicer* species.

Jaccard's dissimilarity indices were calculated for all possible pairs of the nine *Cicer* species (Table 1). Clustering of species, based upon the dissimilarity matrix, is represented by the dendrogram (Fig. 2). The lowest proportion of dissimilarity was observed between *C. reticulatum* and *C. echinospermum*, whereas the highest dissimilarity was found between *C. yamashitae* and *C. cuneatum*. *Cicer arietinum* was most similar to *C. reticulatum*, followed by *C. echinospermum*, *C. pinnatifidum*, *C. bijugum*, *C. judaicum* and *C. yamashitae*, while it was least similar to *C. chorassanicum* and *C. cuneatum* (Table 1). In general, *C. cuneatum* was the least similar to any of the other *Cicer* species.

Ladizinsky and Adler (1975) studied the seed storage protein profiles of seven of the nine annual *Cicer* species (seeds of *C. chorassanicum* and *C. yamashitae* were not available). They observed 6–9 bands in the profile of a single species, whereas 32–37 bands were observed in the present study (Fig. 1). The major difference between the two studies is that both the albumin and globulin fractions were studied in the present study while Ladizinsky and Adler (1975) studied only the albumin fraction. Thus, the present study provides a more detailed profile than the earlier one by Ladizinsky and Adler (1975). The use of the albumin fraction alone as a diagnostic trait for the species and lower taxa has been the subject of great criticism (Jackson et al. 1967; Wolf 1980). The criticism is based on the fact that the variation encountered in the albumin-containing fraction of the total seed proteins might be correspondingly less than variation encountered in the globulin-containing fraction and consequently might be less readily detected. Undoubtedly, when seed-protein electrophoresis is used to study species relationships, it is desirable to have numerous bands since much more taxonomic information is encoded there than in a situation with fewer bands (Boulter 1981). Based on seed-albumin affinities, Ladizinsky and Adler (1975) concluded that *C. arietinum*, *C. reticulatum* and *C. echinospermum* are closely related, and this was confirmed by Vairinhos and Murray (1982, 1983) and also by the pres-

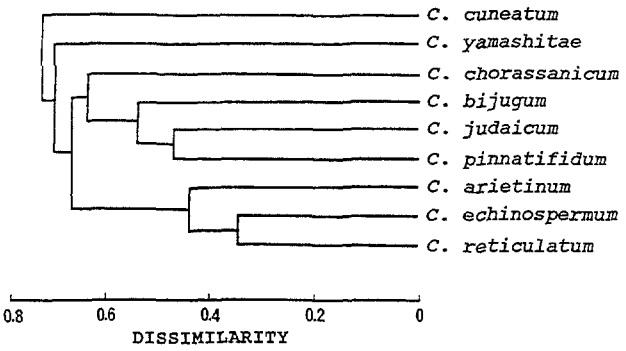

Fig. 2. Dendrogram of the annual *Cicer* species based on dissimilarity matrix of seed storage proteins

Table 1. Dissimilarity matrix, based on the observed seed protein banding patterns, between all possible pairs of the nine annual *Cicer* species

Species[a]	ARI	RET	ECH	JUD	PIN	BIJ	CHO	YAM
RET	0.372							
ECH	0.489	0.342						
JUD	0.615	0.642	0.628					
PIN	0.588	0.691	0.600	0.459				
BIJ	0.612	0.612	0.653	0.511	0.542			
CHO	0.714	0.714	0.727	0.604	0.655	0.600		
YAM	0.660	0.686	0.646	0.673	0.722	0.700	0.673	
CUN	0.714	0.737	0.727	0.679	0.655	0.654	0.679	0.768

[a] ARI, *C. arietinum;* RET, *C. reticulatum;* ECH, *C. echinospermum;* JUD, *C. judaicum;* PIN, *C. pinnatifidum;* BIJ *C. bijugum;* CHO, *C. chorassanicum;* YAM, *C. yamashitae;* CUN, *C. cuneatum*

ent study. However, Ladizinsky and Adler's (1975) findings on the relationships among the other *Cicer* species do not correspond to any other study or to the direct species relationships obtained from genome pairing in known interspecific hybrids.

In view of the fact that only limited information on species relationships, based on genome pairing, is available in the genus *Cicer*, the seed protein profile, which reflects the genetic constitution of the species, should provide some clues. For example, *C. judaicum* and *C. pinnatifidum* have been considered by various botanists to be variants of the same species. Following more detailed morphological comparisons, van der Maesen (1972) decided to keep them as two separate species. This conclusion is strongly supported by the differences in their seed-protein profiles (Fig. 1, Table 1). Of the total 74 bands observed in the profiles of these two species, only 26 could be matched. On the other hand, the close morphological resemblances (excluding seed-coat texture) among *C. arietinum, C. reticulatum* and *C. echinospermum* are supported by their seed-protein profiles.

On the basis of crossability, Ladizinsky and Adler (1976a) grouped seven of the nine annual *Cicer* species into three groups such that interspecific crosses were successful only within a group but not between groups. Group I comprised *C. arietinum, C. echinospermum* and *C. reticulatum,* while Group II included *C. bijugum, C. judaicum* and *C. pinnatifidum.* Group III included only *C. cuneatum.* Recently, on the basis of the two new hybrids, *C. chorassanicum* × *C. pinnatifidum* (Ahmad 1988) and *C. judaicum* × *C. chorassanicum* (Ahmad et al. 1987b), *C. chorassanicum* has been added to Group II, while *C. yamashitae* has been placed in Group IV by itself (Ahmad 1988). Incidentally, the new crossability groups thus formed resemble the clustering of annual *Cicer* species based upon their seed protein profiles (Fig. 2).

The genetic affinities among the annual *Cicer* species determined by the seed-protein profile study here generally corroborate the known genome-pairing data. Thus, in Group I, while *C. arietinum* forms stable hybrids showing eight bivalents regularly with *C. reticulatum,* it differs from *C. echinospermum* by a reciprocal translocation (Ladizinsky and Adler 1976a, b; Ahmad 1988). Similarly, in Group I *C. judaicum* is genomically more closely related to *C. pinnatifidum* and *C. bijugum* (Ladizinsky and Adler 1976a; Ahmad 1988) than to *C. chorassanicum* (Ahmad et al. 1987b). No other interspecific hybrids in the genus *Cicer* are known so far for comparison purposes. On morphological grounds, with its long narrow leaflets, lack of characteristic beak in the seed, presence of tendril and elongated pods, *C. cuneatum* is very different from the other annual *Cicer* species (van der Maesen 1972). These distinct morphological differences between *C. cuneatum* and other annual *Cicer* species are also supported by the comparison of seed-protein profiles.

Ladizinsky and Adler (1975) stated that if the seed-protein profile of the present day cultivated chickpea cultivars was virtually identical with that of the type domesticated thousands of years ago, it was probably also very similar to that of its wild progenitor, and hence the latter should be detectable among the wild species. In the present study, unfortunately, none of the collections of the eight wild annual *Cicer* species examined had identical profiles to that of the cultivated species, and in this respect none can not be regarded as the immediate progenitor of *C. arietinum.* On the basis of seed-protein profiles, *C. reticulatum* was the closest to the cultivated chickpea and, therefore, the most likely candidate for being the wild progenitor of the cultivated species. Such a conclusion is further supported by studies on plant morphology, crossability, genome pairing, isozymes and karyotpyes in the genus *Cicer* (Ladizinsky and Adler 1976a, b; Ahmad et al. 1987a, 1988a; Ahmad 1988). *Cicer echinospermum* is yet another species that comes genetically close to *C. arietinum,* but a reciprocal translocation along with other cryptic structural chromosomal differences between these two species renders the interspecific hybrid either sterile (Ladizinsky and Adler 1976b) or, if partially fertile, leads to hybrid breakdown in the F_2 (Ahmad 1988). Thus, *C. echinospermum* probably did not play a role in the evolution of *C. arietinum.*

The genetic relationships between *C. arietinum* and other annual *Cicer* species lead to speculation that if wild *Cicer* species (other than *C. reticulatum* and *C. echinospermum*) are used for chickpea improvement, relatively more effort would be required to utilize *C. chorassanicum* and *C. cuneatum* than for *C. pinnatifidum, C. judaicum, C. bijugum* and *C. yamashitae.* Of course, valuable interspecific hybrids between *C. arietinum* and the above-mentioned *Cicer* species would have to be first produced, which so far has not been achieved. The useful genetic variability present in *C. echinospermum* and *C. reticulatum* could be utilized with little or no difficulty, respectively, at the present time for the genetic improvement of the cultivated chickpea.

Acknowledgements. The International Crops Research Institute for the Semi-Arid Tropics and the United States Department of Agriculture are gratefully acknowledged for supplying seeds of the wild annual *Cicer* species.

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